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Identification and Characterization of a cDNA Encoding Cytochrome P450 3A from the Fresh Water Teleost Medaka (*Oryzias latipes*)

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A new member of the CYP3A gene family has been cloned from the teleost fish medaka (*Oryzias latipes*) by reverse-transcriptase polymerase chain reaction (RT-PCR). Degenerate primers homologous to highly conserved regions of known CYP3A sequences were used for initial RT-PCRs. Individual PCR products were cloned, sequenced, and identified as those belonging to the cytochrome P450 superfamily based on amino acid sequence similarity and the presence of the highly conserved heme-binding region. PCR products were subsequently used as probes to screen a complementary DNA library. A full-length cDNA clone was identified containing a 1758-base-pair (bp) insert with an open reading frame encoding a single peptide of 500 amino acids. Comparisons of the deduced amino acid sequence to other known cytochrome P450 sequences indicate that this gene product is most similar to the CYP3A gene family and has been designated as CYP3A38 by the cytochrome P450 nomenclature committee. Northern blot analysis identified two abundant CYP3A related transcripts in liver of both male and female adults and demonstrated quantitative differences in abundance according to gender. Similarly, Western blot analysis demonstrated the presence of two abundant cytochrome P450 related proteins in liver of both male and female adults. These results suggests that *O. latipes* contains multiple forms of CYP3A. Heterologous expression of CYP3A38 cDNA in HEK 293 cells produced a single protein that was reactive with anti-scup P450A (CYP3A) polyclonal antibody. Microsomes of HEK 293 cells expressing recombinant CYP3A38 protein actively catalyzed the hydroxylation of testosterone.

Key Words: **medaka; cytochrome P450; CYP3A; steroid metabolism.**

Worldwide, approximately 25,000 species of bony fishes inhabit waters of our biosphere and their composite habitat makes up approximately 70% of Earth's surface. In addition to comprising an important source of human nutrition, a limited number (5 or fewer species) have received appreciable attention as research models. Part of the emphasis for this research has been the fact that the environmental fate of the vast majority of pollutants eventually involves the aquatic medium (associated sediment, water column, or biota). Recent reviews have strongly supported the use of these vertebrates as sentinels for detection of alterations in aquatic environmental quality (1, 2). Medaka fish (*Oryzias latipes*), have been used extensively as a research model for developmental biology, environmental carcinogenesis, and recently for detecting adverse effects of chemicals on reproduction (3, 4). Medaka differ from other fish research models including rainbow trout in that they are a warm-, fresh-, and brackish-water species with a short time to reproductive maturity. Their transparent chorion makes them ideal for developmental studies and breeding pairs can be maintained on a continual 24-h reproductive cycle. Aspects of medaka physiology, development, and genetics are well known. A recent data base has been created documenting isolated genes, transgenic fish, and numerous additional aspects of medaka research (http://bioll.bio.nagoya-u.ac.jp:8000/). As an *in vivo* screening model for suspected carcinogens and environmental pollutants, medaka have shown to be highly sensitive compared to other model fish species includ-

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ing zebrafish (5, 6). Disruption of reproduction, development, and early lifestage mortality are among endpoints observed following exposure of adults and larvae to select xenobiotics (7–9). As development of the medaka model continues, a thorough understanding of the biochemical and molecular actions of environmental pollutants in this species is paramount. To this effect, we have become increasingly interested in examining xenobiotic metabolism and associated cytochrome P450 systems in medaka.

Cytochrome P450s, a large and ubiquitous superfamily of heme proteins, catalyze the oxidative biotransformation of structurally diverse lipophilic compounds including drugs, xenobiotics, and endogenous substances such as fatty acids and steroids. Currently, 12 subfamilies of cytochrome P450 have been identified in fish species including 1(A), 2(B, E, K, M, N, P), 3(A), 4(T), 11, 17, and 19 (10). Their catalytic activities, immunological cross-reactivity and nucleic acid sequences indicate that many of the cytochrome P450 enzymes found in fish species are similar to their mammalian homologs including those responsible for steroid biosynthesis and metabolism.

Cytochrome P450 catalyzed steroid hydroxylation reactions are key to numerous important metabolic pathways including biosynthesis and metabolism of cholesterol, bile acids, vitamin D, prostaglandins, and all major classes of steroid hormones (11). It has been demonstrated that steroid hormones are metabolized by distinct cytochrome P450 enzymes with a high degree of regio- and stereoselectivity (12). Determination of specific metabolic activities for individual cytochrome P450s has been established by various methods including immunoinhibition, chemical inhibition, isozyme specific substrates and activity studies using purified enzyme. More recently, expression of recombinant enzyme and reconstitution systems have enabled the identification of exact metabolic profiles for distinct cytochrome P450 genes (13–16). Through these studies it has been observed that cytochrome P450 3A (CYP3A)[2] gene family members are major contributors to steroid hydroxylation reactions in hepatic microsomes. 6β Hydroxylation of testosterone, progesterone, and androstenedione has been identified as the major catalytic activity of most CYP3A enzymes. To date, the CYP3A subfamily consists of over 43 genes identified from mammalian and nonmammalian species. This subfamily shows a high degree of structural similarity; however, assignment of orthologous sequences has been difficult. Thus all CYP3A genes have been grouped as one subfamily, CYP3A1-43 (17).

CYP3A-like proteins have been purified from teleost species including P450A from scup (*Stenotomus chrysops*), P450b from Atlantic cod (*Gadus morhura*), and P450con/LMC5 from trout (*Oncorhynchus mykiss*) (18–21). The catalytic activities of purified enzyme, immunologic cross-reactivity and inhibition studies strongly suggest that these cytochrome P450 proteins are similar to CYP3A enzymes identified from mammalian species and are likely members of the CYP3A subfamily. While CYP3A-like proteins have been observed in various fish species by immunochemical detection, only one full-length gene sequence and one partial gene sequence have been identified in teleost fish (22). Additionally, to date no information regarding catalytic activities of cloned teleost CYP3A sequences has been demonstrated.

In this study we describe the identification and initial characterization of a new member of the CYP3A gene subfamily from the teleost *O. latipes.* Expression analysis of this CYP3A gene suggests that multiple isozymes are present in liver of adult males and females. Furthermore, we report the catalytic activity of CYP3A38 in heterologous expression studies.

MATERIALS AND METHODS

Fish culture and maintenance. Adult medaka were reared at the Aquatics Center (Institute of Ecology), University of California—Davis, as previously described (23). Briefly, eggs were reared under constant aeration in prefiltered reconstituted water according to EPA guidelines (24). Water was maintained at a constant temperature of 25°C and photoperiod was kept at a constant 16 h light and 8 h dark cycle. Fish were fed a purified casein-based diet developed at Bodega Bay Marine Laboratory, University of California, and modified by us for use with medaka (25).

Reverse-transcribed PCR (RT-PCR). Reverse transcription was carried out with total cellular RNA isolated from adult fish liver. Reverse transcriptase reactions containing 5 μg of total RNA, 0.5 μg oligo(dT) primers, and diethyl pyrocarbonate (DEPC)-treated water (total volume 11 μl) were heated to 70°C for 10 min and quickly chilled on ice. After cooling, 4 μl of 5× reaction buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 10 mM of each deoxynucleotide triphosphate (dNTP), 40 U RNasin (Promega, Madison, WI), and 200 units of Superscript reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) were added to make a total volume of 20 μl. Reactions were incubated at 37°C for 1 h and then heated to 95°C for 5 min to inactivate the reverse transcriptase. Polymerase chain reaction (PCR) was used for second-strand synthesis and subsequent cDNA amplifications. PCR reaction (100 μl) contained 2 μl of the RT reaction mixture as a cDNA template, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10 mM of each deoxynucleotide triphosphate (dNTP) (Amersham Pharmacia Biotech, Piscataway, NJ), 2.5 units of *Taq* DNA polymerase (Promega), and 100 pmol of both up- and downstream degenerate primers. PCR conditions for cDNA amplification with degenerate primers were denaturation at 96°C for 5 min followed by a cycling of annealing at 50°C for 45 s, extension 72°C for 1.5 min, and denaturation 94°C for 45 s for 40 cycles with a temperature ramp of 1°/6 s between the annealing and extension steps. PCR products were analyzed by electrophoresis on 1.5–2.5% agarose gels.

[2] Abbreviations used: CYP3A, cytochrome P450 3A; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; dNTP, deoxynucleotide triphosphate; DIG, digoxigenin; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; UTR, untranslated region; FBS, fetal bovine serum.

Cloning, cDNA isolation, and sequencing. PCR products excised from the agarose gels were purified using QIAEX DNA extraction kit (Qiagen, Valencia, CA), subcloned into T-tailed pCRTMII vector (Invitrogen, Carlsbad, CA) according to manufacturer's protocol, and selected by blue-white screening. Confirmation of PCR product incorporation in plasmids was made by restriction analysis. A ZAP II cDNA library to medaka liver (gift from R. J. Van Beneden, University of Maine) was used for the isolation of a cDNA clone encoding CYP3A38. A 956-bp DNA fragment of medaka CYP3A was generated by PCR and randomly labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech). Approximately 5.0×10^5 plaques were screened through three rounds of isolation. Positive clones were recovered according to the manufacturer's protocol. Clones were subsequently sequenced in both directions using Sp6, T3, T7, or the M13 (−20) primers or by primer walking with sequence-specific oligonucleotides using an ABI prism 377 sequencing unit.

Isolation and analysis of RNA. Total RNA was isolated from adult liver, by the single-step acid guanidinium thiocyanate/phenol method (26). For Northern blot analysis, 1.0 μg total RNA was resolved by electrophoresis through a 1.0% denaturing agarose gel containing 0.66 M formaldehyde and transferred to nylon membranes (Boehringer Mannheim, Indianapolis, IN) by capillary blotting (27). Membranes were prehybridized for 1 h at 68°C in a digoxigenin (DIG) easy-hybridization solution (Boehringer Mannheim). An RNA probe to the CYP3A sequence was labeled using DIG/Genius 4 RNA labeling kit according to the manufacturer's protocol. Approximately 100 ng of probe/ml of hybridization buffer was added and hybridized for 18 h at 68°C. Membranes were washed twice in 2 × SSC (0.15 mM NaCl, 15 mM sodium citrate, pH 7.0) with 0.1% SDS at room temperature for 5 min and twice under higher stringency in 0.1 × SSC with 0.1% SDS at 68°C for 15 min. Probe target hybrids were detected by enzyme-linked immunoassay using alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) and visualized with CDP* chemiluminescent substrate (Boehringer Mannheim).

Extraction and analysis of protein. Total cellular protein was extracted from adult liver by homogenizing 100 mg tissue/ml homogenization buffer containing 50 mM Tris, pH 7.6, 12 mM monothioglycerol, 1.0 mM EDTA, 1.0 mM DTT, 20% glycerol, and 1.0 mM PMSF with a polytron tissue homogenizer. The crude homogenate was centrifuged at 10,000*g* for 30 min followed by 100,000*g* for 1 h. Microsomal pellets were resuspended in homogenization buffer. Protein concentration was measured by the Bradford method (28) with bovine serum albumin as standards. Protein samples were separated by electrophoresis through a denaturing 10% SDS–polyacrylamide gel. After electrophoresis, protein was transferred to nitrocellulose membranes (Boehringer Mannheim) by electrobloting in buffer containing 25 mM Tris–HCl, pH 8.4, 192 mM glycine, and 20% methanol. Membranes were initially blocked in 5% (w/v) nonfat powdered milk in Tris-buffered saline/Tween (20 mM Tris–HCl, pH 8.0, 152 mM NaCl, 0.05% Tween-20). Membranes were then incubated with a primary polyclonal antibody to scup CYP3A, 3.0 μg/1.0 ml 5% (w/v) nonfat powdered milk, followed by washing in Tris-buffered saline/Tween and incubating with alkaline phosphatase conjugated anti-rabbit secondary antibody. Protein targets were visualized with CDP* chemiluminescent substrate (Boehringer Mannheim).

Heterologous expression. For expression of medaka CYP3A38 protein, a full-length cDNA was subcloned from a ZAPII, cDNA library as p-bluescript SK(+/−) by digestion with *Hin*dIII and *Xba*I. The resulting 1758-bp fragment containing the CYP3A38 open reading frame with an additional 34-bp 5′ untranslated region (UTR) and a 221-bp 3′ UTR was ligated into the compatible ends of pCMV5 mammalian expression vector containing cytomegalovirus promoter (obtained from Dr. A. Conley, University California—Davis). The recombinant pCMV5 construct was amplified in DH5α cells (Life Technologies Inc.) and plasmid DNA was isolated/purified using Qiagen plasmid miniprep columns. Human embryonal kidney cells (HEK 293) were grown in D-MEM supplemented with 10% fetal bovine serum (FBS) and 10 mM Hepes at 37°C with 5% CO_2. Next plasmid DNA was transiently transfected into HEK 293 cells using Lipofectamine transfection (Life Technologies Inc.). To achieve this, plasmid DNA (1.5 μg) was added to cells (2.0×10^5 cells/plate) in serum-free media followed by incubation for 6 h at which time media containing 10% FBS was added. Transfected cells were then incubated at 37°C for 48 h, washed, and harvested. HEK 293 cell lysates were prepared by sonication in 250 mM sucrose containing 1 mM EDTA and membrane fractions were prepared by differential centrifugation (final spin 100,000*g* for 60 min). Membrane fractions were resuspended in phosphate buffer containing 100 mM potasium phosphate, pH 7.5, 20% glycerol and 1 mM EDTA. Catalytic activity of microsomal preparations was determined according the method of Waxman and Chang (29). Briefly, incubation mixtures contained 100 mM Hepes, pH 7.4, 0.1 mM EDTA, 250 μg microsomal protein, 50 μM ^{14}C-labeled testosterone and 1 mM NADPH in a total volume of 250 μl. Mixtures were incubated for 1 h at 37°C and then extracted with cold ethyl acetate and testosterone metabolites were analyzed by thin layer chromatography (TLC) on silica gel plates developed in methylene chloride/acetone (4:1). Testosterone metabolites were analyzed by phosphorimaging using a Molecular Dynamics (Sunnyvale, CA) Storm. Individual testosterone metabolites were identified by cochromatography with authentic, unlabeled monohydroxysteroid standards (Steraloids, Inc., Newport, RI) using multiple solvent systems (30). ^{14}C-labeled metabolites were cospotted with each unlabeled standard and developed in solvent systems containing either (A) dichloromethane/acetone (4:1), (B) chloroform/ethyl acetate/100% ethanol (4/1/0.7), or A followed by B. Identification of cytochrome P450 catalyzed testosterone metabolites were made on the basis of comigration with superimposition of both the ^{14}C metabolite and the UV density of the authentic steroid standards. Only when exact superimposition was observed were metabolites positively identified.

RESULTS

Degenerate primer design. Degenerate primers for RT-PCR were designed based on comparisons of conserved amino acid and nucleic acid sequences of known mammalian CYP3A genes. Alignments of CYP3A sequences were compiled using PILEUP (Genetics Computer Group) and revealed several specific regions with high degrees of amino acid similarity. Degenerate oligonucleotide primers encoding these conserved regions were designed as shown in Table I. The appropriate nucleotide sequences were chosen according to the degree of amino acid conservation at each position in the lineup. All codon combinations were considered for each amino acid position in the primer design. Inosine was substituted in instances in which several nucleotides could be used in any one position. This occurred only in the "third base" position. Based on this design, two N-terminal (sense) primers and two C-terminal (antisense) primers were constructed.

Amplification, isolation, and sequencing of cDNA fragments. RT-PCR using degenerate primers designed to amplify conserved regions of CYP3A genes, produced cDNA products of predicted size from adult male and female medaka. Total RNA isolated from adult liver samples was reverse transcribed and used as template for PCR reactions. Primer combinations 3A-7686/3A-7688 and 3-A7689/3A-7687 produced over-

TABLE I

Degenerate Oligonucleotide Primers to CYP3A

Oligonucleotide sequence	Protein sequence	Lineup position[a]	Oligo No.
N-terminal primers			
GCIGAGGACCAATGGAA A A T G	AEDEEW K	123–128	3A-7689
CAAAGIATAATATTCATATTC GTC C C T C T T T T	QSIIFIF	299–306	3A-7686
C-terminal primers			
ACCATATCIAAATACTCCAT G GG T	MEYLDMV	354–360	3A-7687
GGAATCTGIGTCTCTTTACA G T T C G T	CKETQIP	368–375	3A-7688

[a] Corresponds to position of amino acids in CYP3A lineup.

lapping products of 528 and 711 base pairs, respectively. Individual PCR products were subsequently cloned and sequenced. By matching overlapping regions of the PCR products, a total combined sequence of 1064 base pairs was identified. Comparisons of the deduced amino acid sequence to known cytochrome P450 sequences indicated a high degree of similarity to the CYP3A subfamily. Confirmation of the sequences as belonging to cytochrome P450 superfamily, however, was based upon identification of the highly conserved heme binding region.

A full length cDNA sequence of medaka CYP3A gene was obtained by screening a medaka liver cDNA library. Approximately 5.0×10^5 plaques were screened through three cycles with a 956-base-pair probe specific to the putative medaka CYP3A sequence. Two positive clones (S3A4 and S3A15) were identified containing inserts $>$1.7 kb by restriction digest. Individual clones were isolated by phagmed recovery and subsequently used for further studies. The complete nucleic acid sequence of each clone was determined without ambiguity on both strands by primer walking. Restriction analysis and sequencing data indicated that the two clones were identical except for an additional thymine residue at nucleotide position 176 in clone S3A4. This additional nucleotide resulted in a shift in the open reading frame that produced a truncated CYP3A38 protein. No other open reading frame was identified in this clone. The cDNA sequence and predicted translation product of clone S3A15, containing a complete open reading frame, is shown in Fig. 1. The cDNA sequence consists of 1758 nucleotides, with a 1503 coding region flanked by 34-bp 5′ and 221-bp 3′ noncoding regions. Several canonical eukaryotic polyadenylation signals were identified between 22 and 225 bp upstream of the poly(A) tail. A single open reading frame was identified, coding for a protein of 500 amino acids with a single ATG initiation codon, a single TAG termination signal, and a calculated molecular mass of 57,381 Da. The deduced amino acid sequence contains a putative heme binding domain around the Cys^{443} residue, characteristic of cytochrome P450 proteins (31).

Comparison of the deduced amino acid sequence of the medaka CYP3A to other known cytochrome P450 proteins was performed using a BLAST search. Results indicate that the medaka sequence is most similar to CYP3A27 from trout (*O. mykiss*), with a 72% amino-acid identity, and highly homologous to mammalian members of the CYP3A subfamily. Figure 2 shows an alignment of the medaka CYP3A sequence with trout and the four mammalian sequences of highest identity from the BLAST search including: dog CYP3A12 (58%), rat CYP3A9 (58%), sheep CYP3A24 (57%), and human CYP3A4 (57%). Medaka CYP3A sequence contains a high degree of similarity to family 2 cytochrome P450 proteins, especially the I helix and the heme binding region. This sequence was submitted to the P450 nomenclature committee and designated as member 38 of the CYP3A subfamily, GenBank Accession No. AF105018.

CYP3A expression. Both Northern and Western blots were performed to estimate the level of CYP3A expression in untreated male and female medaka. Western blot analyses was performed with a polyclonal antibody to scup (*Stenotomus chrysops*) CYP3A shown to cross react across several teleost species (32). Microsomal fractions of noninduced adult male and female medaka liver demonstrated the presence of two abundant immunoreactive proteins with estimated molecular weights of 57 and 58 kDa, indicating that multiple putative forms of CYP3A are recognized by the anti-scup antibody in medaka liver preparations (Fig. 3). A specific gender difference was consistently observed, with males demonstrating a higher intensity of both CYP3A-like proteins than females.

Expression of CYP3A at the transcriptional level was determined by Northern blot hybridization of total RNA extracted from adult male and female medaka liver using clone S3A15 as a full-length cDNA probe. Under conditions of high stringency, the CYP3A38 cDNA probe hybridized to two abundant transcripts of 1.8 and 2.2 kb in uninduced male and female medaka liver (Fig. 4). Expression of two transcripts is consistent with the Western blot data and supports the identification of two CYP3A genes. In each preparation, loadings were made with equal amounts of RNA by visualization and blots were reprobed using a medaka 18S RNA cDNA probe to quantify steady-state level of RNA. Consistently, Northern blots demonstrated a

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TCAACCATCAGACAGGCTTTACCTCTGAGGCAAAATGGGAAACTTTCTCTACTTCTCTGCTGAAACATGGACCCTACTTGTTGCTTTTAT    90
                                        M  G  N  F  L  Y  F  S  A  E  T  W  T  L  L  V  A  F  I    19
CACACTGCTGCTCGTGTATGCTTACTGGCCTTATGGGACGTTTAAGAGATTAGGAATTCCTGGTCCTAAACCAGTTCCCTTTTTTGGCAC   180
  T  L  L  L  V  Y  A  Y  W  P  Y  G  T  F  K  R  L  G  I  P  G  P  K  P  V  P  F  F  G  T    49
AATGCTGGCTTACAGAAAGGGATTCCCTATCTTTGATGAAGAATGCCACAAGAAATATGGAAAAACTTGGGGCATTTATGACGGCCGTCA   270
  M  L  A  Y  R  K  G  F  P  I  F  D  E  E  C  H  K  K  Y  G  K  T  W  G  I  Y  D  G  R  Q    79
GCCAGTGCTGTGCATCACAGATCCTGCCACCATCAAAGCTGTTCTGGTTAAGGAGTGTTACTCTCTCTTCACCAACCGCAGAAATTTCCA   360
  P  V  L  C  I  T  D  P  A  T  I  K  A  V  L  V  K  E  C  Y  S  L  F  T  N  R  R  N  F  H   109
TCTGAATGGGCCGATGTATGATTCTGTGTTCAATGCTGAAGATGATCAGTGGAAGAGGATCCGTAGCGTCCTCTCTCCTTCCTTCACCTC   450
  L  N  G  P  M  Y  D  S  V  F  N  A  E  D  D  Q  W  K  R  I  R  S  V  L  S  P  S  F  T  S   139
AGGAAGGCTCAAAGAGATGTTTGACATCATGAAGCACCATTCTGCTAACCTGGTCAACAGCATGAAGAAGAAGGCAGACAAAGATGAACC   540
  G  R  L  K  E  M  F  D  I  M  K  H  H  S  A  N  L  V  N  S  M  K  K  K  A  D  K  D  E  P   169
ATTGAACCTGAAGGACCACTTTGGTCCGTACAGCATGGATGTGGTCACCAGCACAGCCTTCAGTGTGGACATCGACTCACTGAACAACCC   630
  L  N  L  K  D  H  F  G  P  Y  S  M  D  V  V  T  S  T  A  F  S  V  D  I  D  S  L  N  N  P   199
CTCAGACCCTTTTGTCACCAACATCAAAAAGATGCTCAAGTTTGATTTTCTGAACCCTCTCTTCCTTCTCGTTGCTTTCTTTCCTTTTCT   720
  S  D  P  F  V  T  N  I  K  K  M  L  K  F  D  F  L  N  P  L  F  L  L  V  A  F  F  P  F  L   229
TGGACCCATAATGGAAAAGATGGAGTTGTCTTTTTTTCCTGCATCCGCATTCGACTTCTTTTATTCTGCACTGCAAAAGATTAAGTCTGA   810
  G  P  I  M  E  K  M  E  L  S  F  F  P  A  S  A  F  D  F  F  Y  S  A  L  Q  K  I  K  S  D   259
TCATAAAAACAACAAGCAAAAGAGTCGAATCGACTTCCTGCAGCTGATGATCGACTCCCAGAAGAACAACCAGAATGGCCTGCAGGATAA   900
  H  K  N  N  K  Q  K  S  R  I  D  F  L  Q  L  M  I  D  S  Q  K  N  N  Q  N  G  L  Q  D  K   289
AGGTTTAAGCGATCATGAGATCCTTTCCCAGGCCATGATATTCATCTTTGCTGGCTATGAAACTACCAGCAGTTCTCTGACATTTTTGGC   990
  G  L  S  D  H  E  I  L  S  Q  A  M  I  F  I  F  A  G  Y  E  T  T  S  S  S  L  T  F  L  A   319
TTACAATCTGGCAACAAACCCCGAGGTCATGAAGAAACTGCAGAAAGAGATAGACGCCACCTTCCCTAACAAGGCTCCCATTCAGTACGC  1080
  Y  N  L  A  T  N  P  E  V  M  K  K  L  Q  K  E  I  D  A  T  F  P  N  K  A  P  I  Q  Y  A   349
ACCACTGTTGGAAATGGAGTACCTGGACTCTGTCGTCAATGAATCTCTCAGACTATACCCCATTGCAGCACGTTTGGAGCGTGTGGCCAA  1170
  P  L  L  E  M  E  Y  L  D  S  V  V  N  E  S  L  R  L  Y  P  I  A  A  R  L  E  R  V  A  K   379
GGCAACTGTGGAGGTTAATGGGCTGGTGATTCCAAAAGATATGGTTGTCATGGTCCCCACCTGGCCGATACAAAGAGACCCTGAACTGTG  1260
  A  T  V  E  V  N  G  L  V  I  P  K  D  M  V  V  M  V  P  T  W  P  I  Q  R  D  P  E  L  W   409
GCCTGAACCTGAGAAATTTAAACCTGAGAGATTCAGCAAGGAGACCAAAGAGACTTTTGACCCCTACACATACATGCCATTTGGTGCAGG  1350
  P  E  P  E  K  F  K  P  E  R  F  S  K  E  T  K  E  T  F  D  P  Y  T  Y  M  P  F  G  A  G   439
ACCGAGAAACTGCATTGGAATGAGATTTGCTCTGGTGTCAATGAAACTTGCCTTGGTGGAGATCCTTCAACAATACAGCTTCTCTGTGTG  1440
  P  R  N  C  I  G  M  R  F  A  L  V  S  M  K  L  A  L  V  E  I  L  Q  Q  Y  S  F  S  V  C   469
TAAGGAAACAGAGATCCCCTTCGAATTGGACATCCAAGGCCTTATCGCACCAAAACGACCCATCTTTCTGAAGCTGGTGCCTCGCTCTGC  1530
  K  E  T  E  I  P  F  E  L  D  I  Q  G  L  I  A  P  K  R  P  I  F  L  K  L  V  P  R  S  A   499
ATGCTAGTAAAAATAAAAATAAAAAAGATGAAAGTTTTTCATTTGATTTTAATGGTAATGTAAATATTGTGCATAAAGGCACCATTGAGTA  1620
  C  *                                                                                          500
ACCCCACATTTGGTGGAATATCACTTTATACCACTATAAGTAAAGCAAAAATAAAACTCCAACACAAATAAATAACAAAGATTTTGTTGT  1710

TTGATCTACTTTGTGATGTGTTTCTAATAAATAGCATACCACCTCTTAAAAAAAAAAAAAAAA                              1758
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FIG. 1. Nucleotide and amino acid sequence of medaka CYP3A38. Deduced amino acid sequence from the open reading frame is in single-letter notation under the second letter of each codon. Putative polyadenylation signals AATAAA are underlined.

gender-specific difference in band intensity between the upper and the lower transcripts. Transcripts from female liver show greater abundance in the upper mRNA transcript, whereas males proved more abundant in the lower mRNA transcript.

Heterologous expression. cDNA inserts for clones S3A4 and S3A15 were subcloned into the mammalian expression vector pCMV5 and used to transfect HEK-293 cells. Western blot analysis of HEK cells transfected with pCMV5/S3A15 revealed the expression of a single protein band. Expressed protein was reactive with anti-scup P450A polyclonal antibody and shared a similar electrophoretic mobility on SDS–PAGE gels with a single CYP3A protein identified from immunoblots of medaka liver microsomes (Fig. 5). Maximal expression of CYP3A38 occurred at 48 h after transfection. As expected, CYP3A proteins were not detected in microsomes from HEK cells transfected with pCMV5/S3A4 or pCMV5 vector only.

Steroid hydroxylase activity of medaka microsomes and expressed CYP3A38 protein was evaluated using testosterone as a substrate. The major hydroxylated testosterone metabolites formed with medaka hepatic microsomal preparations are shown in Fig. 6 (lane 5). The metabolites detected were 6β, 16β, 2β, and 2α-OH testosterone by comigration of ^{14}C-labeled steroid metabolites with authentic unlabeled hydroxysteroid standards in multiple TLC solvent systems. Formation of these major testosterone metabolites is in agreement with those previously characterized with medaka hepatic microsomes (6). CYP3A38 cDNA expressed in HEK 293 cells metabolized testosterone at two predominate positions. The major polar metabolites were identified as 6β-OH and 16β-OH testosterone. Metabolism of testosterone at both the 6β and the 16β position was slightly enhanced by the addition of cytochrome P450 reductase to the *in vitro* incubations (Fig. 6, lane 3). Formation of the 16β-OH metabolite was not observed in microsomes from HEK 293 cells transfected with pCMV5/S3A4 or pCMV5 vector alone (Fig. 6, lane 1 and 2), indicating that formation of this product is specific to expressed CYP3A38. Product formation for the 6β-OH testosterone metabolite was most abundant in microsomes prepared from HEK 293 cells transfected with CYP3A38 (Fig. 6, lanes 3 and 4). 6β-OH testosterone was observed however, in treat-

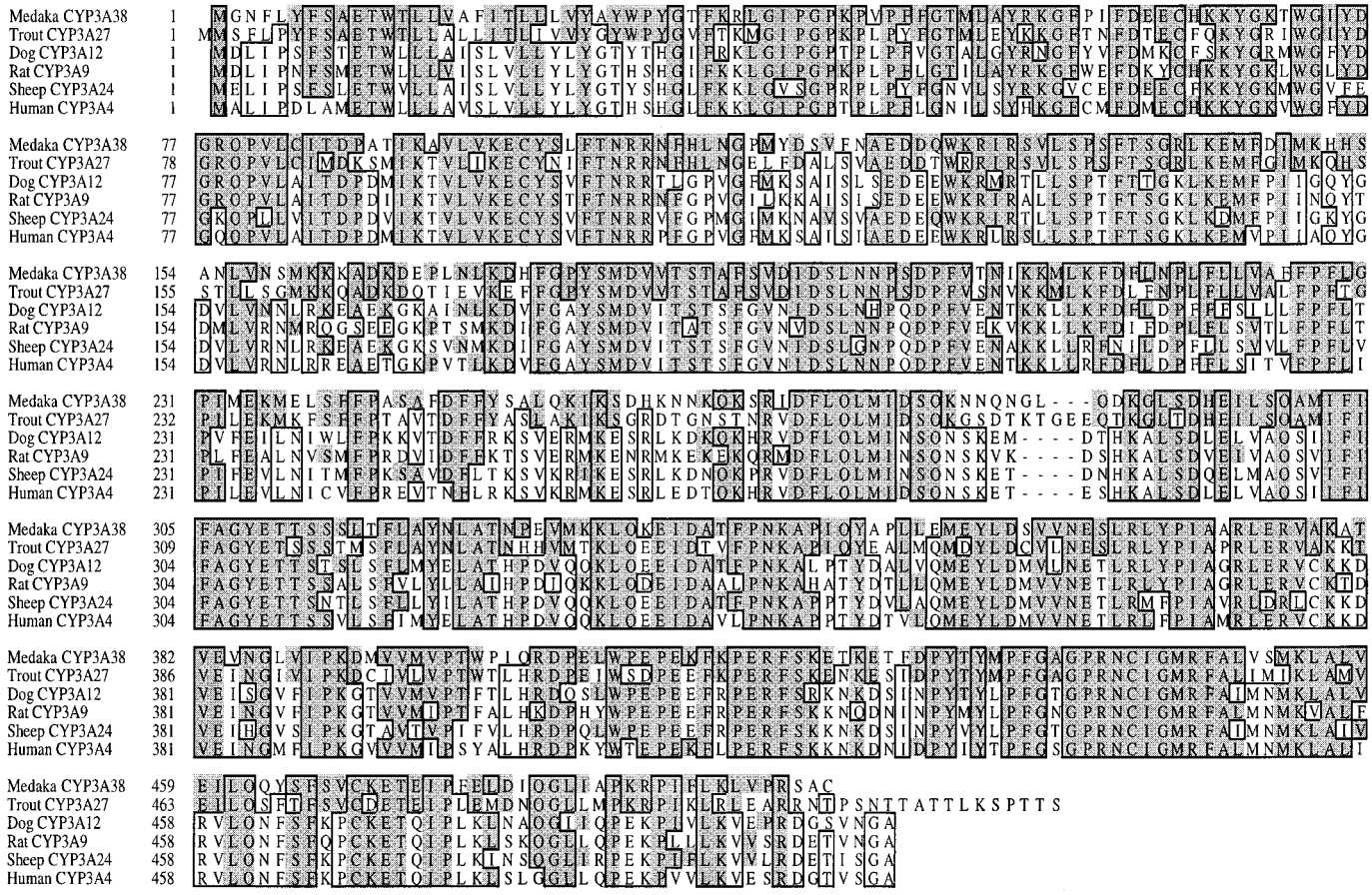

FIG. 2. Alignment of the medaka CYP3A38 sequence with trout CYP3A27, dog CYP3A12, rat CYP3A9, sheep CYP3A24, and human CYP3A4 using SeqVu program V1.0. Boxes represent amino acids that are more than 65% identical. Shaded areas represent amino acids that are identical to the medaka CYP3A38 sequence.

ments containing vector only and pCMV5/S3A4 suggesting that a basal level of testosterone metabolism occurs in HEK 293 cells. Additionally, 2α-OH and 2β-OH testosterone metabolites identified with medaka hepatic microsomes were not observed in HEK 293 cells transfected with pCMV5/S3A15, indicating that CYP3A38 does not actively catalyze formation of these products.

DISCUSSION

Several lines of evidence support our contention that we have cloned a new member of the CYP3A gene family from the fresh water teleost, medaka. Analysis of the deduced amino acid sequence of clone S3A15 displayed a high degree of sequence similarity to members of the cytochrome P450 superfamily of monooxygenases (31). Several well conserved regions of cytochrome P450 are present in the deduced amino acid sequence, including the highly conserved heme binding region, containing Cys^{443}, which acts as the fifth ligand to heme molecule, the aromatic and I helix region, a

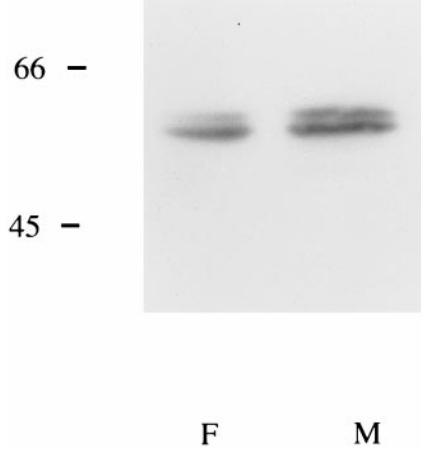

FIG. 3. Western blots of liver microsomes from adult male (M) and female (F) medaka liver. Equal amounts of protein (10 μg each) were applied to each lane and blots were developed with rabbit anti-scup CYP3A antibody. 66- and 45-kDa molecular weight markers are shown on the left-hand side. (Anti-scup CYP3A antibody was a generous gift from M. Celander and J. J. Stegemen.)

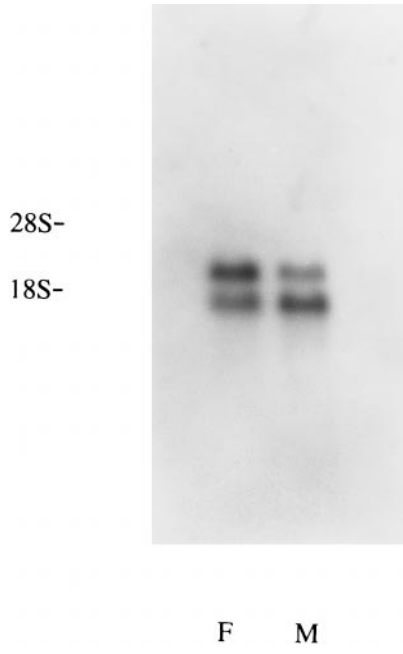

FIG. 4. Northern blot of total RNA (10 μg) isolated form adult male (M) and female (F) medaka livers. Blots were hybridized at 68°C with a DIG labeled RNA probe to the S3A15 CYP3A clone. Size of ribosomal RNA (28S and 18S) indicated on left-hand side.

proline-rich region downstream of the amino terminus, and the signal anchor sequence (33, 34). When compared to cytochrome P450 sequences in the GenBank data base, the deduced amino acid sequence of the medaka gene demonstrates a high degree of similarity to CYP3A orthologs of phylogenetically distant organisms. Within the CYP3A family, medaka CYP3A38 is most similar to trout CYP3A27, suggesting an early phylogenetic lineage of this gene family between two distantly related fish species.

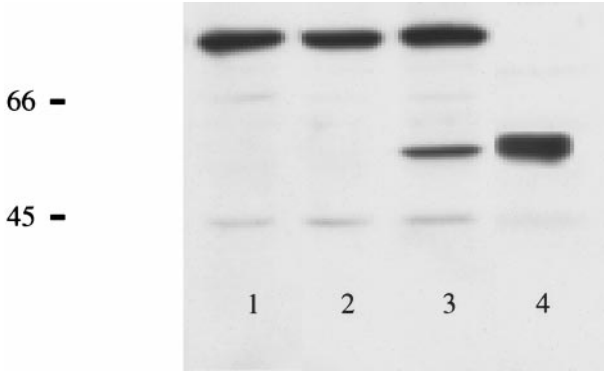

FIG. 5. Western blot probed with polyclonal rabbit anti-scup P450A (CYP3A) antibody to 50 μg microsomal protein of HEK 293 cells transfected with (lane 1) pCMV5 vector alone, (lane 2) pCMV5 vector containing clone S3A4 expressed cDNA, (lane 3) pCMV5 vector containing clone S3A15 expressed cDNA, or (lane 4) 25-μg liver microsomes from adult male medaka shown to contain two abundant 3A bands with the bottom band exhibiting the same electrophoretic mobility as cDNA expressed CYP3A38. 66- and 45-kDa molecular weight markers are shown on the left-hand side.

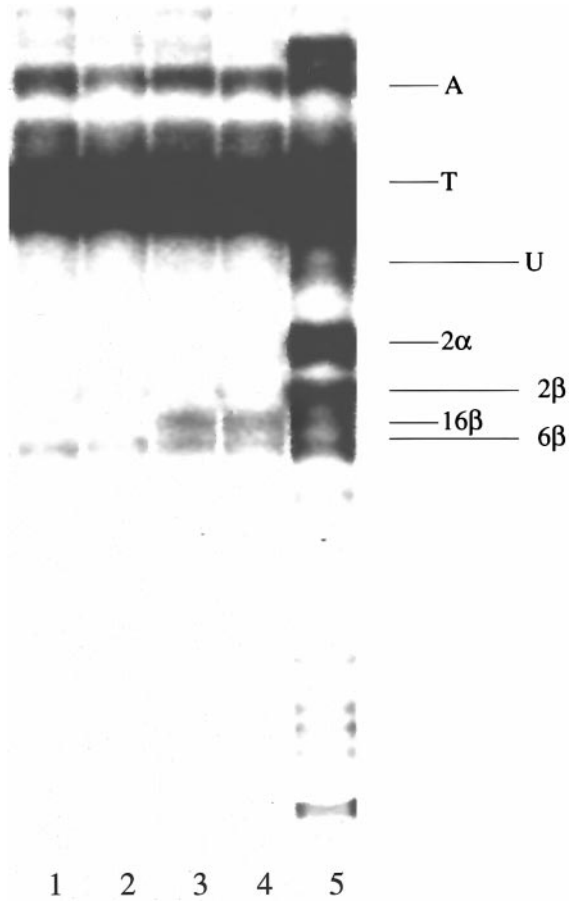

FIG. 6. Autoradiographs of silica gel TLC plates resolving testosterone and respective testosterone metabolites from incubations of HEK 293 cell microsomes transfected with (lane 1) pCMV5 vector alone, (lane 2) pCMV5 vector containing clone S3A4 expressed cDNA, (lane 3) pCMV5 vector containing clone S3A15 expressed cDNA supplemented with purified rat cytochrome P450 reductase, (lane 4) pCMV5 vector containing clone S3A15 expressed cDNA, or (lane 5) liver microsomes of adult male medaka. Individual testosterone metabolites were identified by cochromatography with authentic, unlabeled monohydroxysteroid standards (Steraloids Inc., Newport, RI) using multiple solvent systems (30). Identification of cytochrome P450-catalyzed testosterone metabolites was made on the basis of superimposition of both the ^{14}C metabolite and the UV density of the authentic steroid standards. T, testosterone; A, androstenedione; 6β, 6β-hydroxytestosterone; 16β, 16β-hydroxytestosterone; 2α, 2α-hydroxytestosterone; 2β, 2β-hydroxytestosterone.

Translation of the open reading frame for clone S3A4 containing the additional T residue resulted in a highly truncated 3A protein product of 60 amino acids. The resulting frame shift produced a protein sequence that lost sequence similarity to CYP3A enzymes and lacked functional motifs of cytochrome P450 genes including the heme binding region. Interestingly, Fukada *et al.* (35) reported a similar phenomenon for cytochrome

P450 aromatase isolated from medaka ovarian follicles. Insertion of these additional nucleotides may represent errors in mRNA intron/exon splicing or could be attributed to errors in reverse transcription during cDNA synthesis.

CYP3A gene sequences have been identified in two additional fish species including killifish (*Fundulus heteroclitus*) and rainbow trout. Celander *et al.* (36) reported a 300-bp sequence from killifish and examined phylogenetic relatedness to mammalian CYP3A sequences. Recently Lee *et al.* (22) reported the identification of one full-length CYP3A sequence from rainbow trout. Comparisons of our medaka sequence with those of trout and killifish indicate that marked structural similarities to mammalian CYP3A proteins exist. This is not surprising due to the high degree of cross-reactivity observed between rat, human, and fish CYP3A antibodies (32). The similarity of these teleost CYP3A sequences to mammalian sequences additionally indicates that they may have similar catalytic functions. Miranda *et al.* (37) have shown that microsomal preparations of trout liver catalyze the hydroxylation of testosterone to 6β-hydroxytestosterone. These metabolic oxidations were inhibited by the addition of gestodene, a CYP3A-specific inhibitor. Correlation of 6β-hydroxytestosterone activity in winter flounder was also demonstrated by inhibition of microsomal steroid hydroxylase activity with antibodies to scup CYP3A protein (38).

In mammalian liver, CYP3A isozymes have been shown to catalyze the oxidation of numerous xenobiotics and steroid hormones with stringent stereo- and regiospecificity (39). Expressed CYP3A proteins from several species have demonstrated high affinities toward testosterone, progesterone, androstendione, and other steroids. Predominant metabolic activity for CYP3A genes has been the formation of the 6β-OH metabolite; however, minor metabolites of at positions 1β, 2β, 15β, and 16β have been observed (13–16). In our expression studies we found that expressed CYP3A38 catalyzes the formation of 6β-OH and 16β-OH testosterone. Catalytic activity of expressed CYP3A protein was low in microsomes of CYP3A38 transfected HEK 293 cells and simultaneous addition of purified rat reductase only slightly enhanced metabolite formation. In general, the CYP3A family of enzymes tends to show poor catalytic activity in typical lipid systems that are appropriate for other cytochrome P450 reactions (40, 41). CYP3A mediated oxidations are known to be highly sensitive to assay conditions, including types and amounts of phospholipids, detergents, and cytochrome b5 (40, 42). Although cytochrome b5 significantly enhances CYP3A activity, its effect appears to be substrate dependent (43–45). Alternately, low catalytic activity may represent an incompatability of medaka and other teleost cytochrome P450s with mammalian NADPH reductase and membrane arcitecture. Protein–protein and protein–lipid interactions are important for function microsomal monooxygenase activity. Brian *et al.* (46) observed that CYP3A4, expressed in yeast, did not couple well with NADPH reductase, resulting in inconsistent catalytic activities. Additionally, Yang *et al.* (47) have reported similar low catalytic activities for trout CYP2M1 expressed in COS-1 cells.

Surprisingly, only slight 6β-hydroxylation of testosterone was observed above and beyond background for HEK 293 cells, suggesting that CYP3A38 may be preferential for hydroxylation at the 16β position. 16β-OH in addition to 6β-OH testosterone has been identified as a major metabolite formed by adult liver microsomes from trout (21). 16β-OH has not previously been observed as a major testosterone metabolite in mammalian liver; however, it is an abundant metabolite produced in mammalian kidney and lung (48). Often metabolic properties of different steroids vary with tissue type. Mostly, this has been attributed to selective expression of varying cytochrome P450 genes. However, members of the CYP3A gene family, across and within the same species, demonstrate significant differences in catalytic activity and metabolite profiles. For example, two canine CYP3A isozymes, CYP3A12 and CYP3A26, revealed functional distinctions in steroid hydroxylase activity with CYP3A26 being uniformly less active than CYP3A12 (49). Similar comparisons of human forms CYP3A4, CYP3A5, and CYP3A7 revealed important differences in hydroxylation profiles and catalytic activities using endogenous substrates and a variety of drugs (44, 50).

In this study we have identified multiple CYP3A proteins in medaka liver. This data is consistent with immunoblot analysis of other fish species including tomcod, winter flounder, and trout in which multiple CYP3A-like proteins have been observed (32, 38). It must be noted, however, that multiple transcripts detected by Northern blots may be a result of alternate polyadenylation signaling. Expression of two or more CYP3A isozymes has been observed in numerous species (50–52). Often, however, the functional significance of multiple CYP3A proteins has not been determined. Presumably, isozyme-specific gene expression, substrate specificity, and metabolic profiles among individual CYP3A proteins have relevant physiological significance. For example, fetal forms of CYP3A have been identified in humans and rodents (50, 53). These forms are constitutively expressed during fetal development and diminish remarkably after birth. While important for xenobiotic metabolism, these genes may additionally play an important role in early steroid metabolism and thus fetal development. To date, the physiological role of CYP3A enzymes in medaka is yet to be determined.

This study has resulted in the isolation, expression, and characterization of a cDNA encoding medaka CYP3A enzyme. Functional activity of this enzyme has been identified as 6β and 16β testosterone hydroxylase. This is the first report demonstrating catalytic activity for a CYP3A family member in teleost fish. Immunoblots and Northern blot analysis confirm the presence of multiple CYP3A isozymes in medaka liver with CYP3A38 corresponding to the lower molecular weight protein identified in Western blot studies. Further studies are currently being conducted to examine expression, development, and catalytic activities for each of the medaka cytochrome P450 proteins.

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